Pharmacology Biochemistry & Behavior, Vol. 5, pp. 591–595.

BRIEF COMMUNICATION

Carbachol, Angiotensin-II, Ventricular Spread and Water Balance in Rats[1]

JEFFREY W. PECK

Department of Psychology, University of Utah, Salt Lake City, Utah 84112

(Received 10 May 1976)

PECK, J. W. *Carbachol, angiotensin-II, ventricular spread and water balance in rats.* PHARMAC. BIOCHEM. BEHAV. 5(5) 591–595, 1976. – Injections into the preoptic areas and anterior hypothalamus of as little as 1.0 ng/μl carbachol (5.5×10^{-6} M, 11 pmols total dose) and 0.1 ng/μl angiotensin-II (10^{-7} M, 0.2–0.4 pmols) were dipsogenic or antidiuretic-natriuretic. Lateral ventricular (VL) thresholds for drinking also were 11 pmols for carbachol and 0.2 pmols for angiotensin. The low VL threshold for carbachol supports, without proving, arguments that a limbic cholinergically-coded thirst circuit could reflect leakage form placements near VL to the third ventricle. In contrast, VL thresholds for antidiuresis-natriuresis were 110 pmols for carbachol and 2 pmols for angiotensin. Carbachol was more effective rostral to the paraventricular nucleus and angiotensin dorsal to the supraoptic nucleus. However, lower thresholds in these areas were insufficient to localize receptors, since all cannulas positive for antidiuresis-natriuresis traversed ventricles, and cannulas not traversing ventricles were negative.

Intracranial injection Intracerebroventricular injection Antidiuresis Natriuresis Vasopressin
Cholinergic Preoptic areas Anterior hypothalamus

ROUTTENBERG [12,14] argued that leakage of carbamylcholine chloride (carbachol) to the ventricles from injection or implantation loci probably compromised studies [1, 6, 7, 15] proposing a limbic cholinergically-coded thirst circuit. Drinking followed as little as 0.01–0.2 μg carbachol at points on this proposed circuit [6,7]. Yet, 0.25–4.0 μg of carbachol in the lateral ventricle of rats were not dipsogenic in some studies [1,4], and 0.055 μg was dipsogenic but only with atypically long latency in another [7]. These facts have flawed Routtenberg's argument. The present report reexamined whether carbachol in a lateral ventricle elicited drinking.

In addition, drinking was compared with antidiuresis-natriuresis elicited by ventricular or preoptic-anterior hypothalamic angiotensin-II or carbachol. Peck and Epstein reported [9,11] that the preoptic areas were the sites of the dipsogenic and antidiuretic-natriuretic actions of intracranially-injected angiotensin-II, but Johnson and Epstein [2] showed that spread to the ventricles was necessary for angiotensin-II injected into the preoptic areas to be dipsogenic. Such spread also may have mediated the antidiuretic-natriuretic actions of angiotensin.

METHOD

Animals and Surgery

Six male Long-Evans rats were implanted with one double-barreled intracranial cannula terminating in a lateral ventricle and a second cannula angled to reach the contralateral preoptic areas or anterior hypothalamus without traversing a ventricle. Twenty other rats were implanted with two asymmetrically-placed cannulas aimed for these same regions but introduced perpendicular to the dorsal surface of the cerebral cortex. All rats also had a chronic nasopharyngeal intragastric tube, and 5 of the rats, a catheter in the right jugular vein. These operations have been described [10].

Procedure

Drinking tests. See [10]. Briefly, drinking tests were conducted once weekly. The rats were tested in their home cages, with both food and water present, and were allowed to eat and drink freely after the injectors were inserted, before the test proper began. Injections were not made

[1] Supported by University Research Committee and by USPHS Biomedical Sciences Support Grant RR 07092 to the University of Utah. Thanks to Dr. R. Straight, Director, Medical Research Laboratory, Veterans Administration Hospital, Salt Lake City, Utah for making available the Varian Techtron atomic absorption spectrophotometer, and to J. Denbutter for preparing the histology.

until the rats had assumed a posture indicating sleep, and were made using remote syringes and without disturbing the rats. Injections were unilateral, 2 μl in 18–22 sec. Chemicals were dissolved in isotonic saline (Sodium Chloride Irrigation, Abbott). Tests using angiotensin-II (Hypertensin, Ciba) were completed first. If 1.0 ng/μl (10^{-6}M; 2 μl of solution contained 2 pmols) was dipsogenic, 0.1 ng/μl was tried, then 0.01 ng/μl. When a concentration failed, the last effective concentration was tried; if effective, this concentration now was called the threshold. Only one concentration of angiotensin-II was tried on any test day, so a minimum of 3 tests (3 weeks) were required to establish thresholds. Threshold-finding provided controls for the injection itself causing drinking. First, a dose-response curve resulted; second, an ineffective injection always was bracketed by effective injections differing only in introducing a higher concentration of the dipsogen.

Since rats apparently were sleeping at the time of injection, arousal followed by drinking of any amount within 10 min of the onset of the injection was called a positive response, if repeated on the next test day with the dosage. In negative tests rats did not awaken at all. Rats aroused after injections were observed until they again assumed a posture indicating sleep, although in practice all drinking did occur in one bout interrupted by relatively brief pauses. The duration of drinking reported includes these pauses.

A similar procedure for determining thresholds was followed for carbachol, the initial concentration being either 10 ng/μl (5.5×10^{-5}M; 2 μl contained 110 pmols) or 1.0 ng/μl. The maximum concentrations used were 10 ng/μl (angiotensin) and 100 ng/μl (carbachol).

Analysis of natriuresis and antidiuresis. See [10]. Briefly, rats were hydrated by an initial intragastric injection (12 ml) and sustained infusion (3 ml/100 g BW/hr typically continuing for 3–4 hr) of tap water. The time of each spontaneous void of urine (normally, 10–20 min apart) was recorded and its volume measured. All voids were analyzed for sodium concentration by flame photometry (Varian-Techtron Model AA5). Except for a few instances after termination of drinking tests, tests for antidiuresis-natriuresis were conducted once weekly, 3 days after drinking tests. When water-loading occurred once weekly, rats ate and drank normally and gained weight. More frequent water-loading often led to weight loss. Sodium concentration of the rats' urine 3 days after water-loading was normal.

Both intracranial cannulas were tested and/or several intravenous infusions of vasopressin (Pitressin, Parke-Davis) were made during each test. Thresholds were determined as for the drinking tests. Any increased sodium concentration or decreased urine flow, within the limits of error of collection, could have been a positive response, if repeated on the next test day with that dosage. In practice, only large changes in urine concentration or flow were replicable. Most tests with angiotensin involved repeating the injection 5 min later, which was found necessary for renal changes to result from threshold dosages. The second injection was found unnecessary for carbachol.

Histology. Communication of each lateral ventricular cannula with the ventricle was verified by injection of India ink (2 μl in 19–22 sec) prior to perfusion, after the rats had been anesthetized with pentobarbital. All other cannula placements were verified on frozen and stained sections as described previously [10].

RESULTS

Cannulas terminated in the lateral ventricle (VL) at the rostrocaudal level of the anterior commissure (n = 6), medial preoptic area (POM, n = 3), lateral preoptic area (POL, n = 7), the bed nucleus of the stria terminalis (n = 3), dorsal to or in the anterior hypothalamic area (HA, n = 9), and anteriorly in the lateral hypothalamic area (HL, n = 11). Cannulas showing infection or in brains apparently hydrocephalic on histological examination were omitted.

India ink was found throughout the ipsilateral lateral ventricle for all VL cannulas, but only for 4 of the 6 cannulas was ink also found in the third ventricle. A moderate but not yet gross expansion of the ipsilateral VL of the two exceptional rats suggested the interventricular foramen was blocked, although neither rat appeared ill. These two rats initially responded to angiotensin at the thresholds reported below for the other 4 rats, but by the time of sacrifice neither angiotensin nor carbachol elicited drinking or antidiuresis-natriuresis in either rat. Thus, reports of VL placements are for four rats, the results from the other two VL rats being taken to indicate that communication with the third ventricle was necessary for the effects reported.

As little as 1.0 ng/μl carbachol (2.0 ng total dose, 11 pmols) in VL caused drinking (Table 1). The same threshold, latencies, and volumes drunk were found for preoptic and anterior hypothalamic placements scattered among higher threshold placements (Table 1). There was a dose-response relationship for volume drunk for the latter placements, $t(7) = 2.47$, $p<0.05$. VL rats did not receive 10.0 ng/μl carbachol.

As little as 0.1 ng/μl angiotensin–II (0.2 ng total dose, 0.2 pmols) in VL caused drinking (Table 1). The same threshold and volume drunk was found for preoptic-

TABLE 1

DRINKING CAUSED BY VENTRICULAR OR PREOPTIC-ANTERIOR HYPOTHALAMIC INJECTIONS OF CARBACHOL OR ANGIOTENSIN-II

THRESHOLD (ng/µl)	n	DOSAGE (ng/µl)	LATENCY (sec)	DURATION (sec)	VOLUME (ml)
	LATERAL VENTRICULAR CARBACHOL				
1.0	4	1.0	85±50*	260±133	3.6±0.7
	PREOPTIC-HYPOTHALAMIC CARBACHOL				
1.0	8	1.0	150±122	190±65	3.0±1.3
		10.	60±34	440±108	7.2±2.0
10.	6	10.	205±81	260±173	2.7±1.5
	LATERAL VENTRICULAR ANGIOTENSIN				
0.1	4	0.1	210±128	105±60	1.6±0.6
		1.0	145±60	305±150	3.3±1.4
	PREOPTIC-HYPOTHALAMIC ANGIOTENSIN				
0.1	5	0.1	85±32	140±64	2.6±1.8
		1.0	70±26	240±96	4.2±1.8
1.0	8	1.0	120±44	200±65	2.8±0.8

*Mean ± S.D.

anterior hypothalamic placements scattered among higher threshold placements, but with slightly shorter latencies, $t(7) = 2.0$, $p<0.10$ at 0.1 ng/μl; $t(7) = 2.46$, $p<0.05$ at 1.0 ng/μl. Because the volumes injected were so large (2 μl), the shorter latencies could indicate greater proximity to receptors [17], but could also result from pressure enhancing the effects of angiotensin. There were dose-response relationships for volumes drunk for both VL, $t(3) = 2.39$, $p<0.05$, one-tailed, and preoptic-anterior hypothalamic angiotensin, $t(4) = 2.88$, $p<0.05$, one-tailed.

The retention of urine (6.8 ± 2.0 ml Mean ± SD, 3.7–9.3 range, lasting 40–90 min) and sodium excretion (205 ± 40 μeq, 170–250) following threshold amounts of carbachol did not differ for VL and preoptic-anterior hypothalamic placements, but thresholds did differ. Thresholds for the four good VL cannulas were 10 ng/μl, whereas at 5 cannulas rostral in or dorsal to HA – medial to the fornix and rostral to the paraventricular nucleus near the dorsal plane of the latter – 1.0 ng/μl was effective. Drinking thresholds at these 5 sites were 1.0 ng/μl (n = 2) or 10.0 ng/μl (n = 3). Antiduresis-natriuresis was induced by 10 ng/μl carbachol at 10 other scattered placements.

Volume retention (3.5 ± 1.6 ml, 1.2–6.0, lasting 30–50 min) and sodium excretion (90 ± 85 μeq, 20–310) following threshold amounts of angiotensin–II did not differ for VL and preoptic-anterior hypothalamic placements, but thresholds did differ. VL thresholds were 1.0 ng/μl, whereas at 3 cannulas dorsally in POL at the boundary between POL and HL – directly dorsal to the supraoptic nucleus (as previously reported also [9]) – 0.1 ng/μl was effective. Drinking thresholds at these 3 sites were 0.1 ng/μl (n = 1) and 1.0 ng/μl (n = 2). Antidiuresis-natriuresis was induced by 1.0 ng/μl angiotensin at 11 other scattered placements.

Considering antidiuresis-natriuresis and drinking together, all cannulas at which any dosage of carbachol or angiotensin-II were effective penetrated through a ventricle. At cannulas not traversing a ventricle (n = 12) neither carbachol nor angiotensin-II were effective. Cannulas introduced with slanted trajectories to bypass the lateral ventricle reached HL and POL above or in the supraoptic nucleus (n = 3) and to HA (n = 3). At cannulas introduced with vertical trajectories to these same regions in other rats drinking and antidiuresis-natriuresis had been produced by 10.0 ng/μl carbachol and 1.0 ng/μl angiotensin or lower. No cannulas with slanted trajectory could reach the most medial or the dorsal portions of POM and HA, however.

There were large individual differences, but for each of the 5 rats (6 active cannulas) so tested, intravenous vasopressin infusions matched with intracranial injections for volume retention produced sodium excretions that were similar as well. For angiotensin, 585 ± 95 μU (435–800) – 10–18 μU/100g/min for 6.5–10 min – vasopressin matched threshold intracranial injections; for carbachol, the vasopressin required was 890 ± 175 μU (720-1200) – 8-12 μU/100g/min for 20-32 min. Vasopressin infused initially at a high rate and continued at a low rate more closely mimicked the effects of carbachol than infusion at constant rate. Both intracranial injections and intravenous infusions produced larger natriuresis before water-loading had induced high rates of formation of dilute urine, and lesser or no natriuresis when a high rate of formation of dilute urine had been sustained for some time, or when tests were every other day rather than once weekly, or when tests were a few hours after the operation under ether to implant the jugular catheter. Antidiuresis did not vary in the last 3 conditions.

DISCUSSION

The concentration (1.0 ng/μl) and total dosage (2 ng, 11 pmols) of carbachol that induced rats to drink following lateral ventricular or preoptic-anterior hypothalamic injections in the present study were less than any previously reported in support of a cholinergically-coded limbic thirst circuit (10 ng [6]; 100 pmols [7]). Early studies of the spread of intracranial injections (see [12,15]) assumed largely lateral spread. Johnson and Epstein [2] showed that spread up chronically implanted cannula barrels was a more critical problem. Thus, in the present study carbachol and angiotensin injected through cannulas whose tips were virtually identically placed in the preoptic areas and anterior hypothalamus were dipsogenic if the cannula barrel traversed a ventricle and ineffective if the cannula barrel did not traverse a ventricle. The major weakness of the present study as evidence for ventricular spread and against a cholinergically-coded limbic thirst circuit is that some cannulas yielding low thresholds in previous studies apparently did not penetrate ventricles. Tips of these cannulas were in the corpus callosum (10 ng [6]), hippocampus (10 ng [6]; 100 pmols [7] see below), and, in water-deprived rats only, the cortical nucleus of the amygdala (100 pmols; [7]). However, cannulas reaching these structures closely approached ventricles; injections of carbachol in 0.1 μl volumes (rather than 1μl) into corpus callosum and hippocampus were ineffective ([16], see below).

Previous failures to induce drinking by carbachol placed directly into the lateral ventricle may have resulted from using too much of the drug, so that the side effects reported prevented drinking [1], or from using an insensitive test procedure [4]. Fisher and Levitt [1] reported catatonia, tremors, and "bizarre motor behavior" to accompany implantation of 1–3 μg of crystalline carbachol into the lateral ventricle. Khavari [4] reported that 0.25–4.0 μg of carbachol injected into the lateral ventricle immediately prior to a daily one-hour drinking session of 23-hr deprived rats, did not increase the amount drunk over that normally drunk by the end of the session.

Lovett and Singer [7] found lateral ventricular thresholds of 300 pmols with 6.9 min latency, and reported ventral hippocampal thresholds of 100 pmols with 2.0 min latency as a contrast. Latency for drinking to lateral ventricular carbachol in the present study was 85 sec, well within the normal mean of 2–3 min in the literature. It is difficult to evaluate latencies, since a delay of arousal from apparent sleep in the present study may be less than a delay of recovery from disturbing handling in previous studies (see below). It is possible the long latency following lateral ventricular carbachol seen by Lovett and Singer [7] may have reflected diffusion delays imposed by necrotic material they noted in the ventricle, but not seen in the present study.

As indicated above, a second weakness of the present study as evidence for ventricular spread may be the different method employed. This method deserves comment. Handling rats to insert injectors is an arousing circumstance normally contributing to ingestive behaviors following intracranial injections and normally requiring injections of vehicle to assess. For example, in the present

experiment eating or drinking followed insertion of the injectors in over 70% of all tests, whether or not rats drank to the injections made subsequently. Injections were not made until rats were in a posture normally indicating sleep (not merely quiet but alert rest). For drinking to occur injections of both angiotensin-II and carbachol needed to arouse the rats, and motivate rats to drink rather than eat, since pellets remained in the cage. (Only in 6 tests did rats eat first, including one case for angiotensin where this eating was replicated.)

Thus, although all tests in which drinking of any amount was observed were counted positive, this criterion actually was conservative with respect to the usual methods found in the literature because rats were not already aroused, but aroused only by the injection, and because the drinking occurred again on a second test with that dosage after an intervening negative test at the next lower dosage. On negative tests no arousal at all occurred. Also denying that the low thresholds found in the present study necessarily reflected use of a less demanding criterion than typically employed in the literature is that the mean volume drunk at threshold following carbachol (3.0 or 3.6 ml) equalled the mean volumes drunk reported in the literature once baseline drinking or drinking to injections of the vehicle (which was nil in the present study since rats did not awaken in below threshold tests) were subtracted (e.g., 3.5–4 ml [6], 3 ml [7], 2–2.5 ml [17]).

The present results by no means exclude a limbic cholinergically-coded thirst circuit. But the latencies and thresholds found must provide support for the lateral ventricles mediating the dipsogenic effects of carbachol (and angiotensin) injected into structures adjacent to them, and further for spread from the lateral to the third ventricle for carbachol (and angiotensin) to be dipsogenic. This being stated, it should be noted that thresholds observed by the originator of the ventricular spread hypothesis are themselves inconsistent with these conclusions. In the subfornical organ (SFO), 50 ng carbachol was dipsogenic but 10 ng was not [14]. Furthermore, third ventricular carbachol was ineffective at 100 ng, when lateral hypothalamic carbachol (assumed likely to depend on leakage to SFO) was effective at 100 ng. Nevertheless, thresholds for carbachol injected in 0.1 μl volumes in the medial preoptic-medial septal areas [17] and as assessed by standard technique was identical with the threshold in the present study, although the threshold for angiotensin was somewhat greater (1.25 ng) than for the present study (0.2 ng). This agreement seems all the more significant since the same authors reported hippocampal and corpus callosal placements were insensitive to much higher dosages of carbachol in 0.1 μl volumes [16].

Intracerebroventricular injections of antiotensin-II [3,13] or carbachol [5,8] at higher doses than in the present study have been reported to release antidiuretic hormone (ADH). In addition, because IV vasopressin mimicked the antidiuresis-natriuresis following intracranial injections, the renal responses in the present study apparently depended on ADH.

The lowest thresholds for antidiuresis-natriuresis were as low as those for drinking, although it should be recalled only doses differing by an order of magnitude were tried. However, anterior hypothalamic and lateral preoptic injections elicited antidiuresis-natriuresis with lower thresholds than did lateral ventricular injections, which was not true for drinking. The lowest threshold antiduretic-natriuretic placements were different for carbachol and angiotensin, which makes it unlikely that an artifact dependent upon the volume or rate of injection explains the lower thresholds for preoptic-anterior hypothalamic placements than for ventricular placements.

Nevertheless, the evidence provided by the lowest threshold placements for the location of receptors mediating antidiuretic-natriuretic responses to angiotensin-II and carbachol must be considered weak. Only cannulas traversing a ventricle were positive, and cannulas with slanted trajectories reached some areas (although not all) that were low threshold for cannulas traversing ventricles. Johnson and Epstein [2] showed injection parameters like those in the present study caused spread of antiotensin-II along cannula walls from the preoptic areas to ventricles, and this spread must be assumed to have compromised the present studies. Thus, the previous conclusion by the present author [9,11] that the dipsogenic and antidiuretic effects of angiotensin-II (or carbachol) depend on different receptors seems premature.

REFERENCES

1. Fisher, A. E. and R. A. Levitt. Reply to Routtenberg – Drinking induced by carbachol: Thirst circuit or ventricular modification? *Science* **157:** 839–841, 1967.
2. Johnson, A. K. and A. N. Epstein. The cerebral ventricles as the avenue for the dipsogenic action of intracranial angiotensin. *Brain Res.* **86:** 399–428, 1975.
3. Keil, L. C., J. Summy-Long and W. B. Severs. Release of vasopressin by angiotensin-II. *Endocrinology* **96:** 1063–1065, 1975.
4. Khavari, K. A. Effects of intraventricular administration of carbachol, atropine, and scopolamine on water intake of the rat. *Life Sci.* **7:** 971–977, 1968.
5. Kuhn, E. R. Cholinergic and adrenergic release mechanism for vasopressin in the male rat: A study with injections of neurotransmitters and blocking agents into the third ventricle. *Neuroendocrinology* **16:** 255–264, 1974.
6. Levitt, R. A., C. S. White and D. M. Sander. Dose-response analysis of carbachol-elicited drinking in the rat limbic system. *J. comp. physiol. Psychol.* **72:** 345–350, 1970.
7. Lovett, D. and G. Singer. Ventricular modification of drinking and eating behavior. *Physiol. Behav.* **6:** 23–26, 1971.
8. Miller, N. E. Chemical coding of behavior in the brain. *Science* **148:** 328–338, 1965.
9. Peck, J. W. Discussion: Thirst(s) resulting from bodily water imbalances. In: *Neuropsychology of Thirst,* edited by A. N. Epstein, H. R. Kissileff and E. Stellar. Washington: V. H. Winston, 1973, pp. 99–112.
10. Peck, J. W. and E. M. Blass. Localization of thirst and antidiuretic osmoreceptors by intracranial injections in rats. *Am. J. Physiol.* **228:** 1501–1509, 1975.
11. Peck, J. W. and A. N. Epstein. Antidiuresis following intracranial injections of angiotensin-II into rats. *Fedn Proc.* **30:** 214, 1971, (abst.)
12. Routtenberg, A. Intracranial chemical injection and behavior: A critical review. *Behav. Biol.* **7:** 601–641, 1972.
13. Severs, W. B. and A. E. Daniels-Severs. Effects of angiotensin on the central nervous system. *Pharmac. Rev.* **25:** 415–449, 1973.

14. Simpson, J. B. and A. Routtenberg. The subfornical organ and carbachol-induced drinking. *Brain Res.* **45:** 135–152, 1972.
15. Singer, G. and R. B. Montgomery. Specificity of chemical stimulation of the rat brain and other related issues in the interpretation of chemical stimulation data. *Pharmac. Biochem. Behav.* **1:** 211–221, 1973.
16. Swanson, L. W. and L. G. Sharpe. Centrally induced drinking: comparison of angiotensin II- and carbachol-sensitive sites in rats. *Am. J. Physiol.* **225:** 566–573, 1973.
17. Swanson, L. W., L. G. Sharpe and D. Griffin. Drinking to intracerebral angiotensin II and carbachol: Dose-response relationships and ionic involvement. *Physiol. Behav.* **10:** 595–600, 1973.